Role of spoT-dependent ppGpp accumulation in the survival of light-exposed starved bacteria

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In bacteria, cytoplasmic levels of the effector nucleotide ppGpp are regulated in response to changes in growth conditions. This study describes the involvement of SpoT-mediated ppGpp accumulation in the survival of light-exposed bacteria during fatty acid starvation. In contrast to isogenic wild-type strains and relA mutants, the 'Vibrio angustum' S14 spoT and Escherichia coli relA spoT mutants displayed significant losses in viability in response to cerulenin-induced fatty acid starvation under cool-white fluorescent light. However, when starvation experiments were performed in complete darkness, or under light filtered through a UV-resistant perspex sheet, only a minor decline in viability was observed for the wild-type and mutant strains. This finding indicated that the lethal effect was mediated by weak UV emission. In contrast to the E. coli relA spoT mutant, which lacks ppGpp, the 'V. angustum' S14 spoT mutant exhibited higher ppGpp levels and lower RNA synthesis rates during fatty acid starvation, features that might be correlated with its lethality. In agreement with this finding, fatty acid starvation lethality also occurred upon induction of ppGpp overaccumulation in E. coli. These data suggest that the precise regulation of ppGpp levels in the stressed cell is crucial, and that both the absence and the overaccumulation of ppGpp impair fatty acid starvation survival of light-exposed cells. Moreover, the UV-induced lethal effect during fatty acid starvation was also observed for E. coli strains mutated in rpoS and dps, which, in the wild-type, are regulated directly or indirectly by ppGpp, respectively. The restoration of viability of fatty-acid-starved spoT mutant cells through the addition of exogenous catalase suggested that the observed light-dependent lethal effect was, at least in part, caused by UV-imposed oxidative stress. Based on these results, it is proposed that fatty acid starvation adaptation of light-exposed bacterial cells depends on the development of resistance to UV-induced oxidative stress. This stress resistance was found to require appropriate ppGpp levels, ppGpp-induced RpoS expression and, hence, upregulation of RpoS-regulated stress-defending genes, such as dps.

Keywords: fatty acid starvation, UV radiation

INTRODUCTION

Bacteria in natural environments are constantly challenged by nutrient limitation and stress conditions. Gram-negative, non-differentiating bacteria, such as Escherichia coli and 'Vibrio angustum' S14 (Vibrio sp. CCUG 15956; a model marine bacterium for studying starvation adaptation and stress responses), elicit sophisticated intracellular reorganization programmes in response to nutrient limitation, especially carbon starvation (Kjelleberg et al., 1993; Matin, 1991). Such programmes are characterized by a series of physiological and genetic changes that facilitate the development of multi-stress resistance, and which ultimately lead to long-term survival (Matin, 2000; Srinivasan & Kjelleberg, 1998). Successful starvation adaptation requires many factors, including ppGpp (Cashel et al., 1996) and the stationary phase sigma factor σS (RpoS; Hengge-Aronis, 1996; Loewen & Hengge-Aronis, 1994).

Abbreviations: ppGppase, ppGpp hydrolase; PSI, ribosome-associated ppGpp synthetase; ROS, reactive oxygen species.
ppGpp is an intracellular signal that accumulates in response to nutritional deficiency in prokaryotes, and its accumulation inhibits the synthesis of tRNA and proteins. The metabolism of ppGpp is mediated by two global regulatory proteins, RelA and SpoT (Cashel et al., 1996). RelA is a ribosome-associated ppGpp synthetase (PSI) that facilitates ppGpp accumulation upon amino acid deprivation. The SpoT protein is a bifunctional enzyme, incorporating a weak ppGpp synthetase (PSII) activity and a ppGpp hydrolase (pPGppase) activity. During carbon starvation, the ppGppase activity of SpoT is inhibited, leading to slow rates of ppGpp accumulation (Cashel, 2000). This spoT-dependent ppGpp accumulation occurs within the first few hours of carbon starvation (Flardh et al., 1994), and is essential for subsequent long-term survival (Nystrom, 1994). Furthermore, ppGpp accumulation activates the synthesis of RpoS in E. coli cells (Gentry et al., 1993; Lange et al., 1995), which in turn stimulates the expression of many stress-resistance genes (Loewen et al., 1998). For instance, the RpoS-regulated gene dps (encoding a DNA-binding protein during starvation) has been reported to be important in oxidative stress resistance (Almiron et al., 1992).

While the downstream events of the carbon starvation adaptive response are relatively well understood, little is known about how carbon starvation is sensed by a bacterium and linked to SpoT-mediated ppGpp accumulation and the consequent upregulation of RpoS (Matin, 2000). Studies of the cellular location of SpoT in E. coli have revealed that it is not associated with ribosomes and appears to be a cytosolic protein, which may monitor carbon source availability through a soluble signal molecule (Gentry & Cashel, 1995). Through the use of mutants defective in the fatty acid synthesis pathway and cerulenin, a specific inhibitor of fatty acid synthesis (Omura, 1981), it has been found that fatty acid starvation in the presence of glucose and amino acids stimulates spoT-dependent ppGpp accumulation in E. coli (Seyfzadeh et al., 1993). The signalling pathway leading to this accumulation, and hence the inhibition of rRNA and protein synthesis, did not involve a significant decrease in the synthesis of either phospholipids or ATP. It may well be part of, or identical to, the still elusive SpoT-controlled carbon starvation response mechanism (Seyfzadeh et al., 1993). The precursor of fatty acid synthesis, acetyl CoA, is mainly produced during glycolysis when glucose is present as the sole carbon source. Therefore, glucose deprivation in bacteria apparently causes inhibition of fatty acid synthesis, leading to fatty acid starvation (DiRusso & Nystrom, 1998). Following this rationale, it is possible that SpoT senses a signal generated during fatty acid metabolism to monitor carbon source availability during growth and carbon starvation (DiRusso & Nystrom, 1998). Interestingly, ppGpp has a negative feedback effect on the fatty acid synthesis pathway, by inhibiting glycerol phosphate acyltransferase (Heath et al., 1994).

Fatty acid starvation can be mimicked through the addition of cerulenin, a polypeptide fungal mycotoxin, which binds tightly to two enzymes, 3-oxoacyl-[acyl-carrier-protein] I and II synthases (encoded by fabB and fabF, respectively). This binding prevents the catalysis of the elongation reaction, thus leading to the inhibition of both saturated and unsaturated fatty acid synthesis and hence growth arrest in bacteria (Moche et al., 1999).

Fatty acid starvation halts synthesis of the bacterial lipid membrane, and continued growth of the bacteria results in lethal consequences. Hence, survival of fatty acid starvation depends critically on cessation of cell division and the reduction of growth and net protein synthesis through ppGpp accumulation, together with minimization of existing fatty acid turnover (DiRusso & Nystrom, 1998).

Previous studies in our laboratory have demonstrated that a V. angustum S14 spoT mutant displays a severe loss in viability during carbon starvation (Ostling et al., 1995, 1996), a phenotype similar to that of the E. coli relA spoT mutant (Nystrom, 1994). Moreover, the carbon starvation lethality of the spoT mutant depends on laboratory fluorescent light, which emits extremely weak UV radiation (Ostling et al., 1995). However, ppGpp levels are increased in the carbon-starved V. angustum S14 spoT mutant in contrast to the E. coli relA spoT mutant, which displays decreased levels of ppGpp (Ostling et al., 1995; Xiao et al., 1991). To further address the above postulated link between carbon starvation and fatty acid starvation, two important factors in carbon starvation survival, spoT-dependent ppGpp accumulation and the RpoS regulon, were examined during cerulenin-induced fatty acid starvation. Furthermore, these factors were characterized with regard to their roles in starvation-induced stress resistance. Through the use of various mutant strains of E. coli and V. angustum S14, we report that SpoT-mediated ppGpp accumulation, RpoS and the RpoS-controlled DNA-binding protein (Dps) are required for fatty acid starvation survival in the presence of cool-white fluorescent light. This survival is partly dependent on the resistance to oxidative stress imposed by the weak UV radiation from laboratory fluorescent light. The possible negative effect of ppGpp over-accumulation on fatty acid starvation survival is also discussed.

**METHODS**

**Bacterial strains and plasmids.** The bacterial strains and plasmids used in this study are listed in Table 1. Marine V. angustum S14 (CCUG 15956) strains S141, KF21 and J109 have been described previously (Albertson et al., 1990; Flardh et al., 1994; Marden et al., 1987; Ostling et al., 1991, 1995). E. coli strains MG1655, CF1652, CF1693, CF5747, CF5748, CF4941 and CF4943 were obtained from Michael Cashel (Laboratory of Molecular Genetics, NICHD, National Institutes of Health, USA). E. coli strains ZK126, ZK1000 and ZK1058 were gifts from Roberto Kolter (Department of Microbiology and Molecular Genetics, Harvard Medical School, USA). The plasmid pALS13 (Svitil et al., 1993), carrying a P<sub>lac</sub>-controlled truncated relA gene encoding a 455 aa RelA peptide with PSI activity, was propagated in strain
MG1655, to give strain CF5747. The plasmid pALS14 (Svitil et al., 1993), carrying a P\textsubscript{lac}-controlled controlled truncated relA gene encoding a 331 aa RelA peptide without PSI activity, was propagated in strain MG1655, to give strain CF5748.

**Media and growth conditions.** *E. coli* strains were grown at 30 °C on Luria–Bertani (LB) agar (Sambrook et al., 1989). *V. angustum* S14 strains were grown at 26 °C on VNSS agar (Marden et al., 1985). For liquid cultures of *E. coli* strains, culture side-arm flasks were inoculated with freshly grown colonies and grown at 30 °C on a rotary shaker (160 r.p.m.) in MOPS medium supplemented with 0.2% glucose, 10 µg thiamin ml\(^{-1}\), 40 µg uridine ml\(^{-1}\) and 0.1% Casamino acids (Neidhardt et al., 1974). For liquid cultures of *V. angustum* S14 strains, culture side-arm flasks were inoculated with freshly grown colonies and grown at 26 °C on a rotary shaker (160 r.p.m.) in marine minimal medium (MMM) supplemented with 0.2% glucose, 10 µg thiamin ml\(^{-1}\), 40 µg uridine ml\(^{-1}\) and 0.1% Casamino acids (Ostling et al., 1991). Growth was monitored as a function of optical density measured at 600 nm (OD\textsubscript{600}) with a Pharmacia LKB Novaspec II spectrophotometer. Where appropriate, antibiotics were used at the following concentrations: 100 µg ampicillin ml\(^{-1}\); 12.5 µg tetracycline ml\(^{-1}\); 50 µg kanamycin ml\(^{-1}\) for *E. coli* strains and 85 µg ml\(^{-1}\) for *V. angustum* S14 strains; 50 µg chloramphenicol ml\(^{-1}\); and 200 µg streptomycin ml\(^{-1}\).

**Starvation conditions and light source.** Fatty acid starvation was achieved by adding cerulenin (Sigma) to a final concentration of 200 µg ml\(^{-1}\) to cultures at OD\textsubscript{600} 0.2. Cultures subjected to starvation regimens were stored statically in glass flasks positioned on the laboratory bench top, either directly under the light of a cool-white fluorescent lamp (F15T8CW, Philips Lighting) or under the light covered by a sheet of UV-cutoff perspex (GE Lexan XL-10, Cadillac). The distance between the lamp and the flasks was 50 cm. According to the information provided by the manufacturer, the UV emission spectrum of the light source was mainly identical in the UVA (320–400 nm) region with a peak at 366 nm (Fig. 1a). The intensities of UVB (290–320 nm) and UVC (<290 nm) light were much lower than that of the UVA in the cool-white fluorescent light (Cebula et al., 1995). The perspex sheet and the glass flask used in this study were placed into the beam of a visible–UV spectrophotometer (DU640, Beckman), and their

**Fig. 1.** Spectrum of the cool-white fluorescent lamp used in this study (a) and UV–visible light transmission of the perspex (□) and the glass flask (△) used in this study (b).
UV–visible light transmittance spectra in the 250–500 nm range were obtained (Fig. 1b). The perspex sheet was essentially opaque at all wavelengths below 400 nm (UVA, UVB and UVC). The glass flask was opaque to UV wavelengths of 290 nm or less (UVC). Measured by a wavelength UV-meter (UV products) outside the glass flasks, the mean fluence rate used for the cool-white fluorescent light exposure in this study was 0.2 W m\(^{-2}\). In all experiments, non-illuminated flasks covered with aluminium foil were used as controls. Restoration experiments were performed with illuminated starvation-stressed cells, to which 26 U catalase ml\(^{-1}\) (Sigma) was added at 0 h of starvation. Viability was assessed at appropriate time intervals in terms of c.f.u. ml\(^{-1}\) on appropriate agar plates, by the drop-plate method (Hoben & Somsegaran, 1982).

**Measurement of the rates of total RNA synthesis.** The rate of total RNA synthesis was determined as the rate of pulse-labelled radioactive uridine incorporation into trichloroacetic acid (TCA)-insoluble material (Flardh et al., 1992). Aliquots of an experimental culture were labelled with 0.13 mM \([5,6-\text{H}]\text{uridine}\) (37 Ci mmol\(^{-1}\), Amersham). At appropriate intervals, 50 ml of the culture was removed and added to 800 ml ice-cold 10% TCA; the resulting preparations were kept on ice for 1 h prior to collection of the precipitated material on polycarbonate membrane filters (pore size 0.2 \(\mu\)m, Millipore). The precipitates were washed three times with ice-cold 5% TCA and then transferred to scintillation vials (Crown Scientific). Opti-Phase ‘Hi-Safe’3 (Fisher Chemicals) was used as the scintillation cocktail, and the radioactivity was determined with a Packard series 20000 liquid scintillation counter. The rates of uridine incorporation were determined from the slopes of the linear incorporation graphs (Nystrom et al., 1986).

**Measurement of ppGpp.** The method used to assay ppGpp was performed as described by Cashel (1994). Briefly, cells were grown in the appropriate minimal medium with a low phosphate concentration (0.4 mM). When OD\(_{600}\) 0.05 was reached, \([\text{32P}]\text{orthophosphate}\) (Amersham) was added to a culture aliquot at a concentration of 100 \(\mu\)Ci ml\(^{-1}\), and incubation was continued for about 120 min. Cell densities were monitored in an unlabelled culture aliquot. Sampling for ppGpp began at OD\(_{600}\) 0.2–0.5. The samples were then mixed with 13 M formic acid and three freeze-thaw cycles were performed. Afterwards, the samples were centrifuged and 5 \(\mu\)l samples of supernatant were subjected to one-dimensional TLC on polyethyleneimine sheets (Merck) and developed with 1:5 M potassium phosphate (pH 3.4). Nucleotide abundance was quantified by a Phosphor-Imager Analysis System (Bio-Rad). Amounts of ppGpp were expressed as the total activity (c.p.m.) of blank-corrected ppGpp per OD\(_{600}\) unit of culture.

**RESULTS**

**Light-exposed ‘V. angustum’ S14 spoT and E. coli relA spoT mutants display severe losses in viability during fatty acid starvation**

It has previously been found that fatty acid starvation, induced by the addition of cerulenin in the presence of glucose and amino acids, stimulates spoT-dependent ppGpp accumulation in E. coli (Seyfzadeh et al., 1993). To further investigate the consequences of this ppGpp accumulation in response to fatty acid starvation in bacteria, the survival abilities of E. coli and ‘V. angustum’ S14 relA mutants and spoT mutants were compared to those of their respective isogenic wild-type strains during fatty acid starvation (Fig. 2). After 55 h fatty acid starvation under cool-white fluorescent light, both the ‘V. angustum’ S14 spoT mutant and the E. coli relA spoT mutant displayed a pronounced loss in viability (6.6 and 4.1 log, respectively) in comparison to the wild-type strains and the relA mutants (Fig. 2). Interestingly, these data also demonstrated that ‘V. angustum’ S14 strains are more sensitive to fatty acid starvation than E. coli strains (Fig. 2). When the same starvation experiments were performed in complete darkness, much smaller losses in viability were observed, for all strains tested, in comparison to those exposed to light (Fig. 2). When the same starvation experiments were performed under the fluorescent light filtered by a UV-resistant perspex sheet, only minor losses in viability were observed for all strains (data not shown), which were similar to those observed in the dark (Fig. 2). These results demonstrate that the spoT, but not the relA mutations, in ‘V. angustum’ S14 and E. coli exacerbate the decline in viability during fatty acid starvation in the light. This spoT-dependent increased rate of loss in viability is dependent on the UV emission from the normal cool-white fluorescent lamp. In this experiment, no significant loss in viability was observed in all strains tested under the control conditions (without fatty acid starvation in the light and dark) within 55 h (data not shown). These results specifically relate the lethal effect of light on the spoT mutants to fatty acid starvation, as opposed to any implication of lethality due solely to light exposure. After 5 d treatment in the light or dark, nearly all of the fatty-acid-starved cells of the tested strains were dead, whereas the non-starved cells maintained viability (data not shown), confirming the final lethal consequences of fatty acid starvation.

**The ‘V. angustum’ S14 spoT mutant displays high ppGpp levels during fatty acid starvation**

While both the ‘V. angustum’ S14 spoT mutant and the E. coli relA spoT mutant exhibited light-dependent fatty acid starvation lethality, the alterations in their respective ppGpp levels were distinct during fatty acid starvation (Fig. 3). Wild-type E. coli and ‘V. angustum’ S14 displayed an initial increase in ppGpp levels upon fatty acid starvation in the presence of glucose and amino acids, followed by a gradual decrease in the ppGpp levels after 1 h starvation (Fig. 3). The ‘V. angustum’ S14 spoT strain J109 displayed a threefold higher level of ppGpp in comparison to that of the isogenic wild-type strain S141 after 1 h fatty acid starvation, whereas the E. coli ppGpp\(^*\) strain CF1693 did not display ppGpp accumulation during fatty acid starvation (Fig. 3). In contrast, the relA mutants displayed the same alterations in ppGpp levels as those of the wild-type strains (Fig. 3). The higher ppGpp level observed in the ‘V. angustum’ S14 spoT mutant during fatty acid starvation is similar to that observed during carbon starvation (Ostling et al., 1995). Furthermore, an inverse correlation was observed between ppGpp levels and the synthesis rates of stable RNA during the fatty
Overaccumulation of ppGpp in E. coli causes fatty acid starvation lethality for light-exposed cells

The fatty acid starvation lethality of the E. coli ppGpp<sup>+</sup> strain CF1693 demonstrated that ppGpp is required for survival under light. On the other hand, the higher ppGpp levels and the severe loss in viability of the 'V. angustum' S14 spoT mutant during fatty acid starvation implied that overaccumulation of ppGpp also impairs successful resistance. To further investigate this hypothesis, E. coli RelA overexpression strains CF5747 and CF5748 (Svitil et al., 1993) were assayed for fatty acid starvation survival during light exposure. IPTG-induced RelA overexpression in strain CF5747 elicited ppGpp overaccumulation: a 20-fold increase in the ppGpp levels of the IPTG-treated cells in comparison to a threefold increase in the untreated cells was observed after 1 h fatty acid starvation (Fig. 4a). This overaccumulation of ppGpp reduced the synthesis rates of total RNA (data not shown) and impaired the ability of the cells to survive fatty acid starvation in the light. A 3·2 log drop in the c.f.u. count of the IPTG-treated cells in comparison to a 0·2 log drop in the c.f.u. count of the untreated cells after 40 h fatty acid starvation was observed (Fig. 4b). In contrast, the overexpression of an inactive 331 aa RelA peptide in strain CF5748 (Svitil et al., 1993) had no such effect (data not shown). The 455 aa truncated RelA peptide expressed in strain CF5747 is

Fig. 2. Survival of 'V. angustum' S14 strains and E. coli strains under cool white fluorescent light (■) or in complete darkness (■) during fatty acid starvation. (a) Wild-type 'V. angustum' S141, (b) 'V. angustum' S14 relA mutant KF21, (c) 'V. angustum' S14 spoT mutant J109, (d) E. coli wild-type MG1655, (e) E. coli relA mutant CF1652, and (f) E. coli relA spoT mutant CF1693. Incubation was continued for 55 h subsequent to growth arrest when the c.f.u. counts were approximately 10<sup>8</sup> cells ml<sup>−1</sup> (see Methods for details). Representative data from one of three replicate experiments are shown.

Fig. 3. Alterations in the ppGpp levels during fatty acid starvation under light in strains of 'V. angustum' S14 and E. coli. (a) □, 'V. angustum' S141 wild-type; △, 'V. angustum' KF21 (ΔrelA::kan); ○, 'V. angustum' J109 (spaT::kan). (b) □, E. coli MG1655 wild-type; △, E. coli CF1652 (ΔrelA::kan); ○, E. coli CF1693 (ΔrelA::kan ΔspaT::cam). Cellular ppGpp was labelled with [32P]orthophosphate, extracted and quantified as c.p.m. per OD<sub>600</sub> unit by TLC. See Methods for details. Representative data from one of three replicate experiments are shown.

acid starvation conditions described above (data not shown), corroborating previous reports that have demonstrated RNA synthesis rate measurements to reflect variation in ppGpp levels (Cashel et al., 1996).
metabolically unstable, which avoids the toxic effect of protein overexpression (Svitil et al., 1993). Taking strain CF5748 as a negative control, the data suggest that the light-dependent fatty acid starvation lethality of strain CF5747 may be due to ppGpp overaccumulation, rather than to the toxic effects of protein overexpression, plasmid maintenance, or IPTG itself. Moreover, the artificial overproduction of ppGpp resulted in reduced lethality in strain CF5747 in the absence of starvation, as well as during starvation in the dark (data not shown). In E. coli, the spoT203 allele has been found to reduce ppGpp degradative activity, resulting in an eightfold elevation of ppGpp levels (Sarubbi et al., 1988). During fatty acid starvation under light exposure, E. coli CF4943, containing the spoT203 mutation, displayed a significant decline in viability in comparison to its isogenic parent strain, CF4941 (Fig. 4c). These results support our hypothesis that overaccumulation of ppGpp impairs the survival ability of the illuminated fatty-acid-starved cells.

Light-exposed E. coli strains with mutations in rpoS and dps display severe losses in viability during fatty acid starvation

It has been found that ppGpp upregulates the synthesis of the stationary-phase sigma factor RpoS in E. coli (Gentry et al., 1993; Lange et al., 1995). RpoS and RpoS-regulated proteins (e.g. Dps) are crucial for starvation-induced stress resistance and starvation survival (Almiron et al., 1992; Eisenstark et al., 1996; Hengge-Aronis, 1996; McCann et al., 1991). To investigate the role of the RpoS regulon in fatty acid starvation, rpoS and dps mutant E. coli strains were compared in survival ability to the isogenic wild-type strain during fatty acid starvation (Fig. 5). After 55 h fatty acid starvation under cool-white fluorescent light, both mutants displayed significantly greater losses in viability in comparison to the wild-type strain. The rpoS mutant exhibited a 2-9 log decrease in viability, whereas the corresponding values for the dps mutant and the wild-type strain were 5-6 log and 0-5 log, respectively (Fig. 5). When these starvation experiments were performed in complete darkness, much smaller losses in viability were observed for all strains tested in comparison to those kept in the light (Fig. 5). When these starvation experiments were performed under the fluorescent light filtered through a UV-resistant perspex sheet, the minor losses in viability, for all strains, were identical to those observed in the dark (data not shown). These data indicated that rpoS and the RpoS-regulated stress-resistance gene dps are involved in fatty acid starvation survival during light exposure. The main challenge to the cells under these conditions appears to be the UV radiation from the cool-white fluorescent lamp used. Moreover, the difference between the losses in viability of the rpoS and dps mutants during fatty acid starvation suggests a potential direct role for DNA-binding protein in starvation (Dps) in fatty acid starvation survival under light.

Addition of catalase to the illuminated culture largely restores viability of ‘V. angustum’ S14 and E. coli spoT mutants during fatty acid starvation

The main fraction of the UV emission from the cool-white fluorescent light is UVA radiation (Cebula et al., 1995), which causes oxidative damage to DNA and proteins in bacteria through photo-oxidation (Eisenstark, 1989). Therefore, the light-dependent lethality of the spoT mutants during fatty acid starvation is likely to be related to the UV-induced oxidative stress. To address the role of oxidative stress resistance in the fatty acid starvation response, we investigated the effect of catalase, an H2O2-degrading enzyme, on the survival of the fatty-acid-starved E. coli and ‘V. angustum’ S14 cells. Addition of catalase resulted in an increase in viability for illuminated spoT mutant cultures of ‘V. angustum’ S14 and E. coli that were fatty acid starved for 35 h, in
Fig. 6. Effect of catalase on the survival of illuminated fatty acid starved cells of ‘V. angustum’ S14 and E. coli. (a) ‘V. angustum’ S14 wild-type (squares) and ‘V. angustum’ J109 (spoT::kan) (circles). (b) E. coli MG1655 wild-type (squares) and E. coli CF1693 (ΔrelA::kan ΔspoT::cam) (circles). Experiments were performed with illuminated fatty-acid-starved cells in non-supplemented medium (solid symbols) or medium supplemented with catalase (open symbols). Representative data from one of three replicate experiments are shown.

comparison to non-catalase-supplemented cells (Fig. 6). Specifically, the ‘V. angustum’ S14 spoT mutant displayed an increase in viability of three orders of magnitude (Fig. 6a), whereas the E. coli relA spoT
mutant displayed an increase of two orders of magnitude (Fig. 6b) upon the addition of catalase. The c.f.u. counts of the ‘V. angustum’ S14 and E. coli wild-type strains were also increased slightly by catalase addition after 35 h of starvation (Fig. 6), indicating that catalase has a general effect on the fatty acid starvation survival of the illuminated cells. These results suggest that the UV-imposed oxidative stress accounts for the severe decline in viability of the fatty-acid-starved spoT mutant cells.

**DISCUSSION**

It has been reported that fatty acid starvation in the presence of glucose and amino acids stimulates a spoT-dependent ppGpp accumulation in E. coli (Seyfzadeh et al., 1993). The present report demonstrates that a ‘V. angustum’ S14 spoT mutant and an E. coli relA spoT mutant display severe losses in viability in response to cerulenin-induced fatty acid starvation under cool-white fluorescent light in the presence of glucose and amino acids (Table 2). In contrast, the respective relA mutants and wild-type strains exhibited little or no loss in viability under the same conditions. On the other hand, the mutant cells starved in the dark or under the light filtered by a UV-cutoff perspex sheet did not display this pronounced loss in viability. Therefore, the spoT-dependent increased rate of decline in cell viability during fatty acid starvation is provoked by fluorescent light illumination, in particular the weak UV emission from the light used. This light-dependent lethal effect during fatty acid starvation also occurred in the E. coli strains with mutations in rpoS and dps (Table 2).

**Role of SpoT during carbon and fatty acid starvation**

We propose that SpoT senses a signal generated in fatty acid metabolism to monitor carbon source availability during growth, and during carbon starvation. The SpoT-mediated ppGpp accumulation (Seyfzadeh et al., 1993) and the spoT-dependent survival during fatty acid starvation are similar to the characteristics of the carbon-starved cells (Flardh et al., 1994; Nystrom, 1994; Ostling et al., 1995; Xiao et al., 1991). These data suggest that the signalling pathway leading to ppGpp accumulation upon fatty acid starvation may be part of, or identical to, the as yet elusive SpoT-controlled carbon starvation response mechanism (Seyfzadeh et al., 1993). Consistent with this idea, it was found that carbon-starved E. coli cells with a mutation in accD (a gene encoding acetyl-CoA carboxylase, a key enzyme in fatty acid biosynthesis) display an aberrant pattern of ppGpp accumulation, excessive cell division, and progressive failure to respond rapidly to a nutritional up-shift (Seyfzadeh, 1994). It is well known that the precursor of fatty acid synthesis, acetyl-CoA, is mainly produced during glycolysis when glucose acts as the sole carbon source. Therefore, glucose deprivation in bacteria apparently causes inhibition of fatty acid synthesis, leading to fatty acid starvation (DiRusso & Nystrom, 1998). Following this rationale, it is possible that SpoT normally responds to a signal produced in fatty acid metabolism to monitor carbon source abundance, and optimizes growth economy of the cell accordingly (DiRusso & Nystrom, 1998; Gentry & Cashel, 1995). This putative signal may be synthesized from fatty acid derivatives, such as fatty acyl-CoA, and inhibit the SpoT

**Table 2. Relative viability of the strains in fatty acid starvation**

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<thead>
<tr>
<th>Strain</th>
<th>Fatty acid starvation*</th>
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<tr>
<td></td>
<td>Light</td>
</tr>
<tr>
<td>‘V. angustum’ S14 wild-type</td>
<td>++</td>
</tr>
<tr>
<td>‘V. angustum’ S14 wild-type + catalase</td>
<td>++</td>
</tr>
<tr>
<td>‘V. angustum’ S14 relA mutant</td>
<td>+</td>
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<tr>
<td>‘V. angustum’ S14 spoT mutant</td>
<td>–</td>
</tr>
<tr>
<td>‘V. angustum’ S14 spoT mutant + catalase</td>
<td>+</td>
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<tr>
<td>E. coli wild-type</td>
<td>++</td>
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<tr>
<td>E. coli wild-type + catalase</td>
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<tr>
<td>E. coli relA mutant</td>
<td>+</td>
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<tr>
<td>E. coli relA spoT mutant</td>
<td>–</td>
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<tr>
<td>E. coli relA spoT mutant + catalase</td>
<td>+</td>
</tr>
<tr>
<td>E. coli rpoS mutant</td>
<td>–</td>
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<tr>
<td>E. coli dps mutant</td>
<td>–</td>
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<tr>
<td>E. coli wild-type P lac::relA</td>
<td>++</td>
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<tr>
<td>E. coli wild-type P lac::relA + 1 mM IPTG</td>
<td>–</td>
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* + + +, < 0.2 log loss; + +, 0.5–1 log loss; +, 1–2 log loss; –, 2–3 log loss; ---, 3–4 log loss; -- -- --, > 4 log loss.
function responsible for ppGpp accumulation and further starvation survival, perhaps by directly binding to the SpoT protein or by binding to another positive factor required for SpoT function (Seyfzadeh et al., 1993). Hence, fatty acid starvation leads to the loss of this signal and upregulation of SpoT-mediated ppGpp accumulation. Alternatively, carbon starvation and fatty acid starvation may use independent signal transduction mechanisms to trigger spoT-dependent ppGpp accumulation and further starvation adaptive responses. Further studies of the signalling pathway(s) leading to ppGpp accumulation during fatty acid starvation are currently being undertaken.

UV-induced oxidative stress and fatty acid starvation

It is interesting that the fatty acid starvation lethality of the cells with mutations in spoT, rpoS and dps is provoked by standard laboratory cool-white fluorescent light. Furthermore, this lethal effect was found to be mostly due to the weak UV emission of the light. According to the information provided by the manufacturer, the main fraction of the UV emission from the cool-white fluorescent lamp is UVA radiation (Fig. 1a). The lamp also emits very low levels of UVB and UVC that may cause direct damage to DNA (Cebula et al., 1995). However, the effect of UV can be ruled out in this study since the glass flasks we used block UVC (200 nm–320 nm), thus the experiments were performed with UVA and UVB (320 nm–400 nm). UVA and UVB produce many reactive oxygen species (ROS) through photo-oxidation and hence cause oxidative damage to DNA, proteins and membrane lipids in bacteria (Eisenstark, 1989). ROS are produced during normal cellular metabolism, but can be augmented after cellular exposure to UV, especially in the presence of various photo-sensitizers (Jagger, 1981). The light-dependent lethal effects reported here are suggested to be mainly due to UV-induced oxidative stress rather than to direct DNA damage. This hypothesis was also supported by the following findings: (i) the spoT mutants were found to be more sensitive to UV radiation as well as to H2O2 exposure in comparison to their isogenic parents during fatty acid starvation (unpublished data); (ii) addition of exogenous catalase to the growth medium during fatty acid starvation (unpublished data); (iii) addition of exogenous catalase to the growth medium during fatty acid starvation reversed most of the loss in cell viability of the spoT mutants provoked by fluorescent light illumination (Fig. 6). We note that since only UVA and UVB of solar UV irradiation reach marine surface waters, the natural habitats of marine Vibrio species, our findings have clear ecological implications (Joux et al., 1999). Moreover, our observed lethal effects occur in starved cells exposed to extremely weak UV radiation from laboratory cool-white fluorescent light. Its intensity (0.2 W m−2) is much lower than that of full sunlight (33 W m−2), and is typical of the intensities found in marine surface waters, where the UV radiation is quenched (Jeffrey et al., 1996).

One of the factors that appear to play an important role in the response of bacterial cells to UV irradiation is the mechanism known as stringent response. It is well known that UV exposure affects tRNA containing a 4-thiouridine modified base, which cross-links with cytidine, losing its charge capacity and triggering the stringent response with RelA-mediated ppGpp accumulation and the consequent arrest of tRNA and protein biosynthesis. This mechanism induces a growth delay, including impairment of replication and membrane integrity in E. coli (Jagger, 1981; Thiam & Favre, 1984), Salmonella typhimurium (Kramer et al., 1988) and Pseudomonas aeruginosa (Fernandez & Pizarro, 1999). It may be questioned whether the UV emission employed in this study, rather than fatty acid starvation, provoke the ppGpp accumulation observed in this study. However, our experimental conditions are distinct from the UV radiation used in the studies reported above. Firstly, the fluence rate of UV emission used here is 0.2 W m−2, which is much lower than the levels reported before: 216 W m−2 for E. coli (Jagger, 1981; Thiam & Favre, 1984), and 35 W m−2 for S. typhimurium (Kramer et al., 1988) and P. aeruginosa (Fernandez & Pizarro, 1999). Secondly, in contrast to the findings reported in the papers cited above, we did not observe growth delay or significant ppGpp accumulation in the growing cells illuminated by the cool-white fluorescent light (data not shown). Thirdly, the patterns of ppGpp accumulation we observed for the fatty-acid-starved cells in the light and dark are similar (data not shown), suggesting that starvation, instead of light, is needed as a means of inducing ppGpp. It is therefore suggested that the extremely weak UV emission used in this study do not serve as a factor to trigger RelA-mediated ppGpp accumulation in the starvation adaptive response, but act as a stress challenge to the starved cells.

SpoT-mediated ppGpp accumulation has been shown to be important for carbon starvation survival (Nyström, 1994; Ostling et al., 1995). The stationary-phase sigma factor RpoS is required for resistance to nutrient starvation, UVA radiation and oxidative stress (Eisenstark, 1998; Eisenstark et al., 1996; Hengge-Aronis, 1996; Loewen & Hengge-Aronis, 1994). The RpoS-regulated protein Dps (DNA-binding protein in starvation) is also known to be important in oxidative stress resistance, via protection of DNA against ROS attack (Almiron et al., 1992). Hence, the light-dependent lethality of the spoT, rpoS and dps mutants during fatty acid starvation may not be surprising. However, it is worthwhile to note the differences among the losses in viability during fatty acid starvation for the relA spoT mutant, the rpoS mutant and the dps mutant. The greater lethality for the relA spoT mutant in comparison to that for the rpoS mutant can be explained by the recent finding that ppGpp is required for the expression of RpoS-controlled genes in a RpoS-independent manner (Kvint et al., 2000). Furthermore, McGlynn & Lloyd (2000) demonstrated that ppGpp plays a direct role in promoting the ability of E. coli cells to survive UV-induced DNA damage. This increased survival may be explained by the destabilization of RNA polymerase promoter open complexes, or by stalled elongation complexes (Bartlett et al., 1998; Zhou & Jin, 1998), thereby minimizing stalled RNA polymerase blocks to
replication fork progression and excision repair. The greater lethality of the *dps* mutant in comparison to that of the *rpoS* mutant indicates that Dps may play a more direct role in protecting cells against stress attack in starvation. This assumption is also supported by the multi-factorial regulation of Dps expression, i.e. it is activated by OxyR during exponential phase and by IHF and RpoS in stationary phase (Altuvia *et al*., 1994). Together, these results suggest that the fatty acid starvation adaptation of the illuminated bacterial cells requires successful defence against UV-imposed oxidative stress, and that the starvation-induced stress resistance is dependent on *spoT*-dependent ppGpp accumulation, as well as the upregulation of *rpoS* and RpoS-controlled stress resistance genes such as *dps*.

**Accumulation of ppGpp and fatty acid starvation survival**

The light-exposed *E. coli* *relA spoT* mutant CF1693 loses cell viability and fails to accumulate ppGpp following fatty acid starvation. However, the light-exposed *E. coli* *relA* mutant maintains cell viability and accumulates ppGpp as the wild-type strain does during fatty acid starvation. This suggests a correlation between the loss in viability and the lack of *spoT*-dependent ppGpp accumulation. A similar correlation has been found in carbon starvation adaptation, in which ppGpp accumulation during the first few hours of starvation has a long-term effect on the survival ability for many days (Matin, 2000; Nystrom, 1994). The fatty acid starvation lethality of the *E. coli* *relA spoT* deletion mutant is not surprising, since this ppGpp- starved strain is more sensitive to many other stresses, including carbon starvation and amino acid starvation (Nystrom, 1994; Xiao *et al*., 1991). The *V. angustum* S14 *spoT* mutant J109 displayed threefold higher ppGpp levels and a greater and prolonged elevation of ppGpp levels during starvation has a long-term effect on the survival ability for many days (Matin, 2000; Nystrom, 1994). The fatty acid starvation lethality of the *E. coli* *relA spoT* deletion mutant is not surprising, since this ppGpp- starved strain is more sensitive to many other stresses, including carbon starvation and amino acid starvation (Nystrom, 1994; Xiao *et al*., 1991). The *V. angustum* S14 *spoT* mutant J109 displayed threefold higher ppGpp levels and a severe loss in viability in comparison to the wild-type strain S14 during fatty acid starvation. J109 contains a *J109* displayed threefold higher ppGpp levels and a significant increase in survival under light. These results support our hypothesis that overaccumulation of ppGpp impairs the survival ability of the illuminated fatty-acid-starved cells. Apirakaramwong *et al*., 1999) reported a similar decline in cell viability in stationary phase due to artificial ppGpp overaccumulation. After overexpression of a recombinant constitutive *relA* in *E. coli*, a rapid increase in the levels of ppGpp was observed, accompanied by a severe loss in viability and a decrease in the RpoS level (Apirakaramwong *et al*., 1999). This finding is unexpected because it has been reported that RpoS synthesis is upregulated by ppGpp (Gentry *et al*., 1993; Lange *et al*., 1995). However, it suggests that the prolonged ppGpp accumulation may become toxic for cells in late-phase starvation through the reduction of RpoS levels. Since accumulation of ppGpp has the dual consequences of stimulating and inhibiting gene expression, it is likely that the proper balance of these effects must be set for an appropriate physiological response. In other words, overaccumulation of ppGpp may inhibit rRNA synthesis and down-regulate the protein synthetic capacity to a level which is below that required for sufficient translation of stress resistance proteins. Although the lethal effect of ppGpp overaccumulation with fatty acid starvation was more significant than that without starvation, the sole killing effect of ppGpp overproduction might not be ruled out, which was supported by recent studies (Aizenman *et al*., 1996; Sat *et al*., 2001) showing that MazEF-mediated programmed cell death is induced by the artificial overproduction of ppGpp, leading to higher concentrations than those of physiological conditions.

Based on the results presented in this study, we propose that successful fatty acid starvation survival of the light-exposed bacterial cells depends on an adaptive response, which requires appropriate *spoT*-dependent ppGpp accumulation, the upregulation of RpoS and hence the induction of RpoS-controlled stress resistance proteins such as Dps. Lack, or overaccumulation, of ppGpp impairs the fatty acid starvation survival of light-exposed cells. These early-phase starvation-induced proteins are able to exert prevention and/or repair of the UV-induced oxidative damage, leading to effective protection against naturally occurring weak UV radiation.

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**REFERENCES**


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